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Ultrastructural Observations of Oogenesis in the Fern *Adiantum flabellulatum* L. (Adiantaceae)

Jian-Guo Cao*, Quan-Xi Wang, and Xi-Ling Dai
College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, China
Jeffrey G. Duckett
School of Biological and Chemical Sciences, University of London, London, UK

Abstract.—Oogenesis in the fern *Adiantum flabellulatum* was studied using transmission electron microscopy. The newly formed egg is closely appressed to the archegonial jacket cells. Plasmodesmata connect the egg and the ventral canal cell, but are absent between the egg and the jacket cells. During maturation, a separation cavity forms around the egg. However, a pore region, persistently connecting the egg and the ventral canal cell, is reported for the first time. Subsequently, an osmiophilic egg envelope is formed, from a combination of the products of sheets of endoplamic reticulum and osmiophilic bodies in the egg cytoplasm. The absence of this envelope from the region where the egg and ventral canal cell were formerly interconnected produces a fertilization pore, which is believed to be an entrance for the sperm penetrating the egg. In the mid stages of egg maturation, the nucleus envelope produces extensive evaginations which then appear to be sequestered into the egg cytoplasm recalling a similar phenomenon in the eggs of other ferns. Degeneration of the neck canal cells is associated with the secretion of copious mucilaginous material.

Key Words.—*Adiantum flabellulatum*, fern, fertilization pore, nuclear evagination, oogenesis

Previous studies on oogenesis in ferns including *Pteridium aquilinum* (L.) Kuhn (Bell and Mühlethaler, 1962a, b; Bell and Duckett, 1976), *Histiopteris incisa* (Thunb.) J.Sm. (Bell, 1980), *Osmunda cinnamomea* var. *asiatica* Fern. (Bao *et al.*, 2003), and *Dryopteris crassirhizoma* Nakai (Bao *et al.*, 2005) have revealed that the egg is surrounded by a conspicuous extra egg 'membrane'. This structure has been regarded as a venter coat covering the top of the egg in *Athyrium filix-femina* (L.) Roth (Fasciati *et al.*, 1994) and *Ceratopteris richardii* Brongn. (Lopez-Smith and Renzaglia, 2008). In addition derived taxa such as *Pteridium aquilinim* (Bell, 1972), *Histiopteris incisa* (Bell, 1980), *Dryopteris crassirhizoma* (Bao *et al.*, 2005) and *Athyrium filix-femina* (Fasciati *et al.*, 1994) produce conspicuous nuclear evaginations during egg maturation. In contrast, this feature appears to be absent in more primitive families (e.g., Marattiaceae, Osmundaceae, Schizaeaceae and Dipteridaceae (Bell, 1986; Bao *et al.*, 2003)). We recently discovered in *Ceratopteris thalictroides* (L.) Brongn. that the extra egg membrane is absent from the central part of the upper egg surface and that this acts as a fertilization pore, through which the spermatozoid penetrates the egg (Cao *et al.*, 2009). This structure in the egg of a sister species, *Ceratopteris richardii*, has been interpreted as a receptive

* Author for correspondence: E-mail: cao101@shnu.edu.cn Tel: +86 21 64322526

site (Lopez-Smith and Renzaglia, 2008). No detailed description about the formation of the structure has been reported; however, whether the fertilization pore exists in other ferns has not been investigated. Here we describe the development of a fertilization pore in oogenesis in *Adiantum flabellulatum* L. (Adiantaceae) and detail other noteworthy features of oogenesis in this fern.

Materials and Methods

Spores of *Adiantum flabellulatum* were collected from plants in Wanmulin Nature Reserve of Fujian province, China. The spores were surface sterilized with 5% sodium hypochlorite solution for 3 min. After rinsing three times with distilled water, the spores were sown on a modified Knop's solution (0.8g $Ca(NO_3)_2 \cdot 4H_2O$; 0.2g KH_2PO_4; 0.2g KNO_3; 0.2g $MgSO_4 \cdot 7H_2O$, dissolved in 1 liter distilled water), solidified with 1.5% agar in culture dishes. These dishes were placed in an artificial climate chamber under conditions of 25°C in the light (18 h) and 20°C in the dark (6 h). After 6 to 7 weeks, archegonia had developed on the lower surface of the gametophytes just behind the growing apex.

Gametophytes bearing archegonia were placed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer at room temperature for 6–12 h. The specimens were subsequently washed three times with the same buffer, postfixed in 2% aqueous osmium tetroxide for 2 h, rinsed three times in buffer and embedded in Spurr's resin (SPI-Chem, USA) via a graded acetone series. Specimens were thick sectioned for the presence of the archegonia and thin-sectioned with a diamond knife on an Ultracut-E ultramicrotome (Reichert-Jung, Germany). The thin sections were stained with uranyl acetate and lead citrate. All specimens were observed with H-600 electron microscope (Hitachi, Japan).

Results

The newly formed egg.—The archegonium of *A. flabellulatum* contains three cells; the egg, the ventral canal cell (VCC), and the neck canal cell (NCC) (Fig. 1A). When the egg is newly formed, the periphery of the three cells is closely appressed to the archegonial jacket cells (Fig. 1A–D). There are numerous plasmodesmata between the egg and the VCC, and also between the VCC and NCC (Fig. 1D, E) but these are absent between the three cells and the jacket cells (Fig. 1C, D). In longitudinal sections the young egg is approximately trapezoidal or quadrate (Fig. 1A, B). The transverse diameter of the young egg is about 25μm and its height is about 15–17μm (Fig. 1A, B). The nucleus of the young egg is roughly spherical and typical sections show one or two nucleoli (Fig. 1B). The chromatin of the egg cell is slightly more dispersed than that in the VCC (Fig. 1B). The newly formed egg contains abundant vesicles, distributed principally around its periphery and base (Fig. 1B) and plastids with large starch grains and few lamellae (Fig. 1C). Mitochondria are distributed evenly throughout the cytoplasm of the egg (Fig. 1B, C). At this

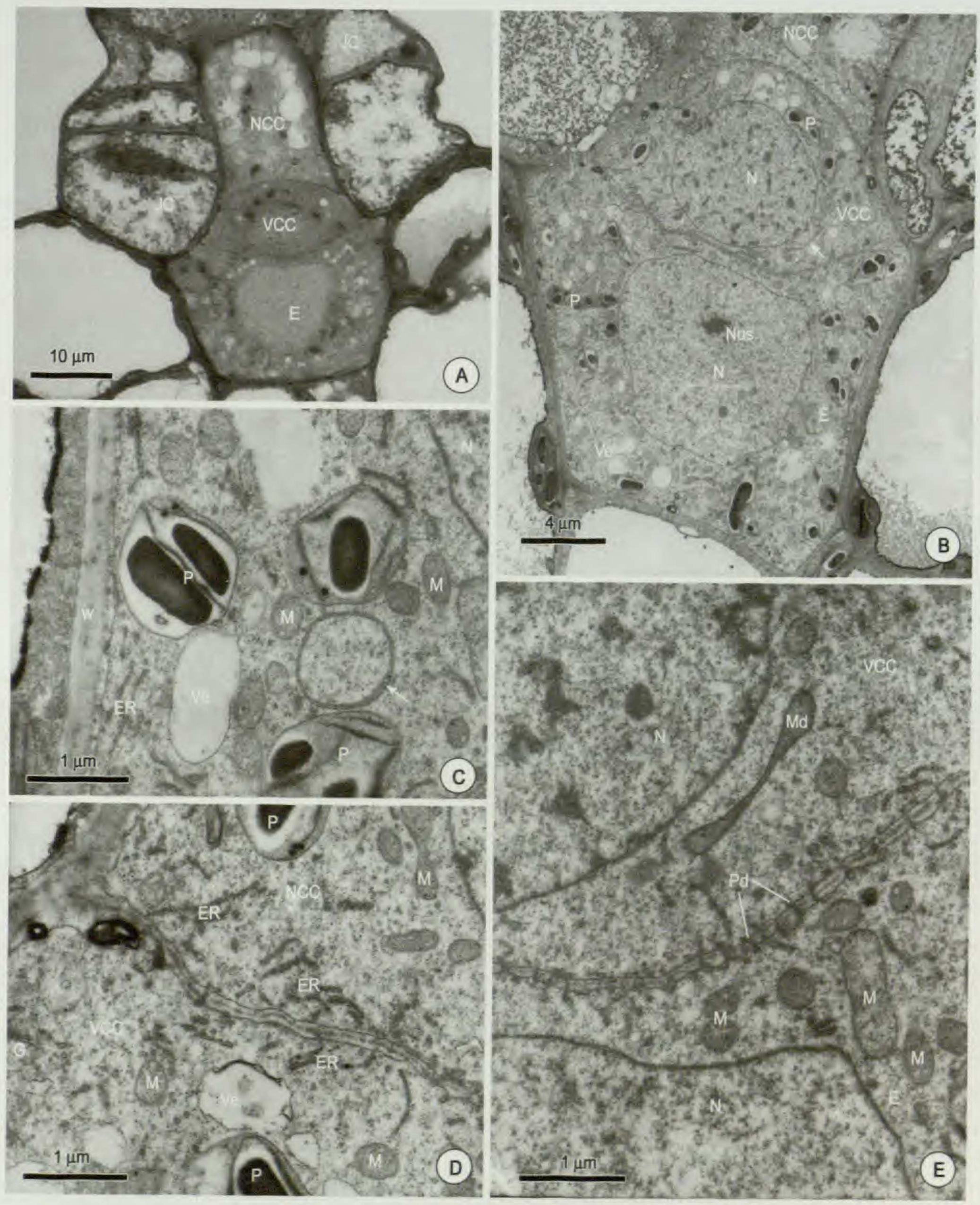

FIG. 1. Young archegonium of *Adiantum flabellulatum*. **A.** An archegonium, containing a young egg (E), a ventral canal cell (VCC) and a neck canal cell (NCC) (under LM, all other figures are observed under TEM). **B.** An archegonium, containing a young egg (E), a VCC and a NCC. Arrow indicates a dumbbell-shaped mitochondrion in the VCC. **C.** Magnification of Fig. 1B, showing the organelles in the egg cell. Arrow indicates a sac-like mitochondrion. **D.** Part of the VCC and NCC showing the organelles. **E.** Plasmodesmata (Pd) connecting the egg and the VCC. A dumbbell-shaped mitochondrion (Md) lies beside the nucleus of the VCC. ER, endoplasmic reticulum; G, Golgi bodies; M, mitochondrion; N, Nucleus; Nus, nucleolus; P, plastid.

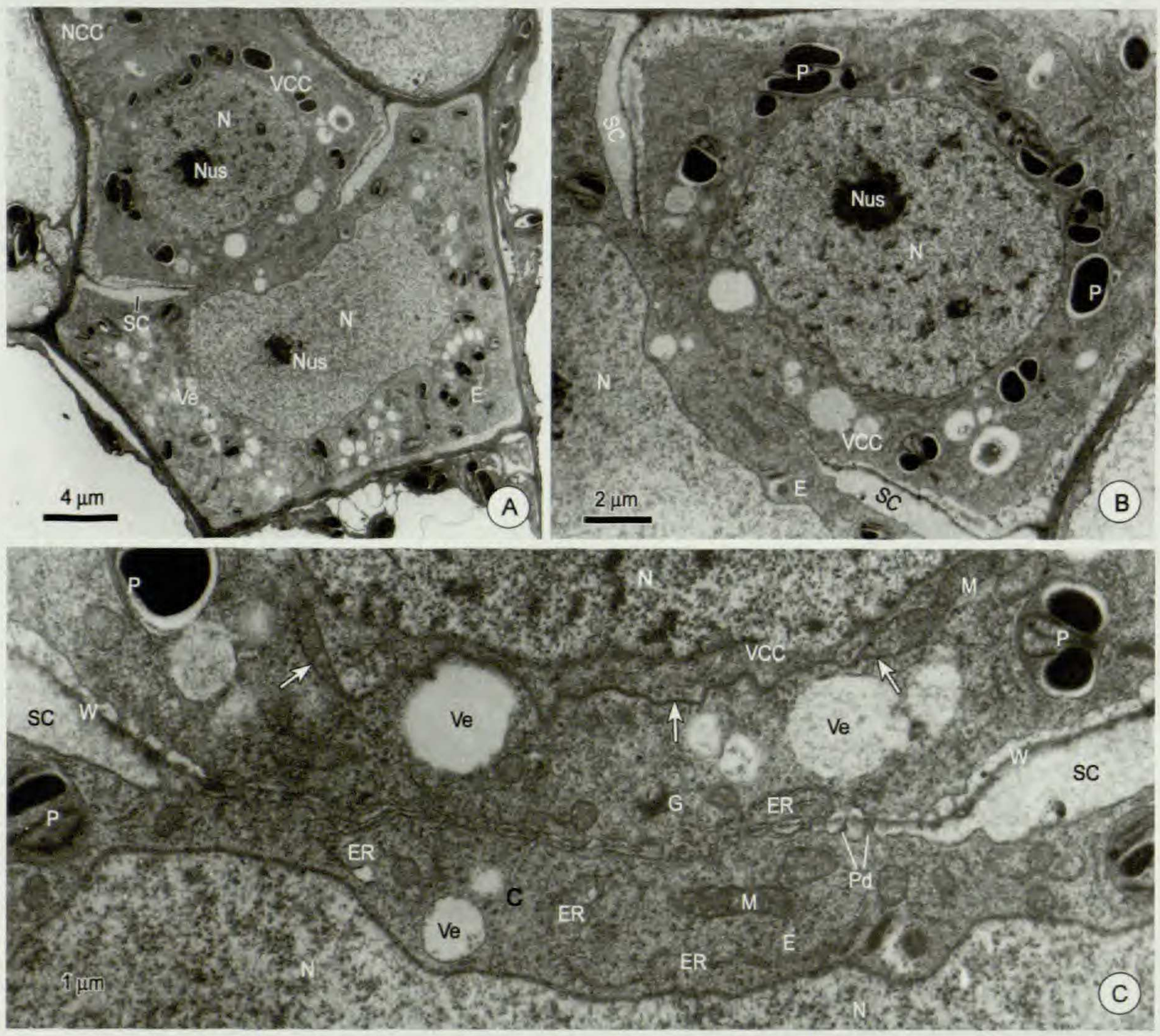

FIG. 2. Separation cavity formation. **A.** An archegonium, containing a maturing egg (E), a VCC and a NCC. A separation cavity (SC) has formed around the periphery of the egg. **B.** The VCC, showing the asymmetrical distribution of the organelles. **C.** Central region between the egg and the VCC, showing plasmodesmata (Pd) that connect the egg and the VCC; a sheet-like mitochondrion (arrows) lies beneath the nucleus of the VCC. E, Egg; ER, endoplasmic reticulum; G, Golgi body; M, mitochondria; N, Nucleus; Nus, nucleolus. P, plastid; Ve, vesicles; W, cell wall.

early stage the cytological features of the VCC, the NCC and the egg are closely similar (Fig. 1D) apart from the occurrence of dumbbell-shaped mitochondria in the lower lateral parts of the VCC (Fig. 1B, arrow; Fig. 1E).

Egg maturation.—Egg maturation is associated with substantial cytological changes including the formation of a separation cavity, an osmiophilic egg envelope, a fertilization pore and nuclear evaginations.

Formation of the separation cavity.—The separation cavity initially begins to form around the periphery of the upper surface of the egg and then extends around the lower part of the egg (Fig. 2A–C). However, the egg remains connected to the VCC by well- developed plasmodesmata at the central region in its upper surface (Fig. 2C). As the egg develops, the separation cavity above the egg expands centripetally and the connection region is correspondingly

reduced to a final diameter of about 2.7 μm (Fig. 3A, D). Plasmodesmata remain in the pore region (Fig. 3D). The nucleus now becomes somewhat irregular in outline with a depressed upper surface (Fig. 3A).

At this stage, the eggs contain single sheets of endoplasmic reticula (ER) often lying parallel to the plasmalemma (Fig. 3C). The egg plasmalemma becomes increasingly dark-stained except in the pore region (Fig. 3D) and dentate around the periphery (Fig. 3C, arrowhead). Vesicles containing osmiophilic materials are often present beneath the pore region (Fig. 3D) and in the cytoplasm of the VCC (Fig. 3B, arrows).

Organelles in the VCC are typically asymmetrically distributed. Plastids are clustered around the nucleus and are absent from the lower part of the cell (Fig. 2B), which contains vesicles, mitochondria and Golgi bodies (Fig. 2B, C; Fig. 3D). A large sheet-like mitochondrion lies beneath the nucleus of the VCC (Fig. 2E; Fig. 3D).

Formation of the osmiophilic egg envelope and the fertilization pore.—The second conspicuous feature of the maturing egg is the formation of a layer of osmiophilic egg envelope (formerly called 'extra egg membrane') around the egg (Fig. 4A). At this time parallel sheets of ER appear next to the lower surface of the egg (Fig. 4B), osmiophilic globules are visible next to the egg envelope (Fig. 4C, arrows), and the upper egg envelope is thicker than the lower part (Fig. 4B, C). The pore region still connects the egg cell with the VCC (Fig. 4A, D) and no osmiophilic egg envelope is formed in this region (Fig. 4D).

The organelles in the egg cytoplasm differ greatly to those in the previous stage; vesicles have almost disappeared, as has starch from the plastids (Fig. 4D, E), and mitochondria are increasingly prominent (Fig. 4B). In the VCC, vesicles and Golgi bodies increase above the fertilization pore (Fig. 4C, D), whilst mucilaginous material accumulates around this cell (Fig. 4C).

Nuclear evaginations.—During formation of the osmiophilic egg envelope, the original ellipsoidal nucleus of the egg changes into a cup-shape (Fig. 4A) and its surface produces numerous sac-like evaginations (Fig. 4E and F, Nev). These are usually between 0.5–0.7 μm in diameter and remain connected to the main body of the nucleus via narrow isthmuses (Fig. 4E, arrow). Sacs similar to the nuclear evaginations, but without connection to the nucleus in sections, occur the cytoplasm of the egg (Fig. 4F, arrows). The matrix of the evaginations resembles that of the main body of the nucleus (Fig. 4E, F).

The mature egg.—By the time the egg is mature the greatly shrunken VCC is surrounded by copious mucilaginous material, but still remains connected to the egg via the fertilization pore (Fig. 5A). Clusters of plastoglobuli have replaced starch in the VCC plastids (Fig. 5B). Parallel sheets of rough ER accumulate in the upper cytoplasm of the egg (Fig. 5 C, D) and evaginations remain numerous around the lower surface of the nucleus (Fig. 5C, F).

The transverse diameter of the fertilization pore almost reaches 2.8 μm (Fig. 5D). The membranes around the pore seem to be interwoven to form the thick border around the pore (Fig. 5D). Sometimes the vesicles containing osmiophilic material can be seen within and outside of the fertilization pore (Fig. 5D, Veo). The osmiophilic globules, as seen in previous stage, are also

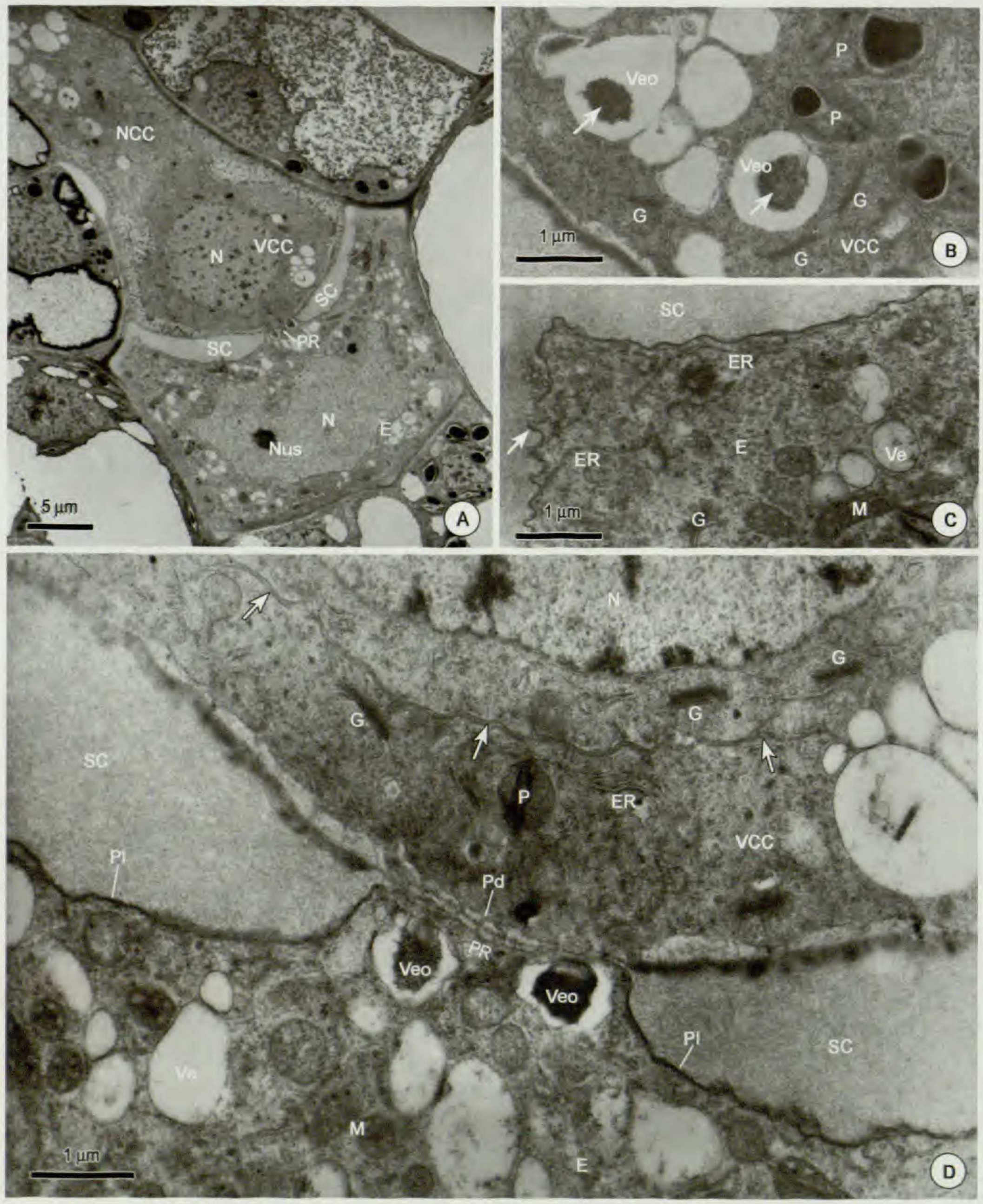

FIG. 3. Initiation of the fertilization pore. **A.** An archegonium containing a maturing egg (E), a VCC and a NCC. A pore region (PR) persistently connects the egg and VCC. The nucleus of the egg becomes slightly cup-shaped. **B.** Cytoplasm of the VCC showing the vesicles often containing the osmiophilic materials (Veo). **C.** Upper-side part of the egg, showing the dentate surface. **D.** Magnification of Fig. 3A. showing well- developed plasmodesmata (Pd) in the pore region. Vesicles containing the osmiophilic materials (Veo) below the pore region. A sheet-like mitochondrion (arrows) lies beneath the nucleus of the VCC; Golgi bodies (G), ERs and vesicles (Ve) are abundant in the cytoplasm of the VCC. M, mitochondria; N, Nucleus; Nus, nucleolus. P, plastid; Pl, plasmalemma; SC, separation cavity.

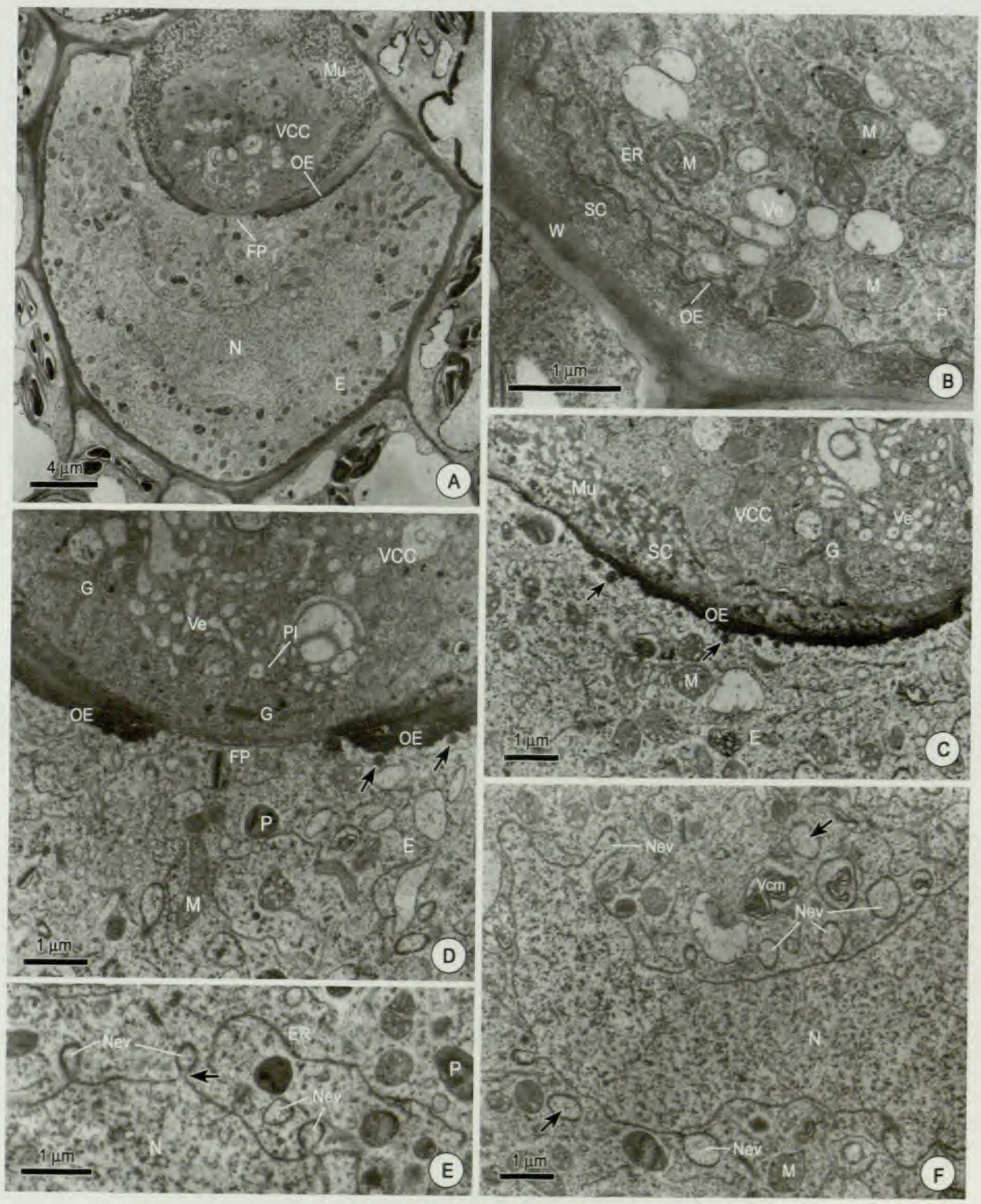

FIG. 4. The fertilization pore and nuclear evaginations. **A.** The egg cell is surrounded by a lay of osmiophilic envelope (OE). Fertilization pore (FP) on the upper surface of the egg. **B.** Lower part of the egg, showing the organelles. **C.** The osmiophilic egg envelope (OE) in the upper surface of the egg. Osmiophilic globules (arrows) are often seen next to the egg envelope. **D.** Section through the FP, the OE is absent in this region. Arrows indicate the osmiophilic glubles. **E.** Nuclear evaginations (Nev) usually connected to the main body of the nucleus by a narrow isthmus (arrow). **F.** Some of the evaginations are probably pinched off of the nucleus (arrows). E, Egg; M, mitochondria; N, Nucleus; Mu, mucilaginous materials; P, plastid; SC, separation cavity.

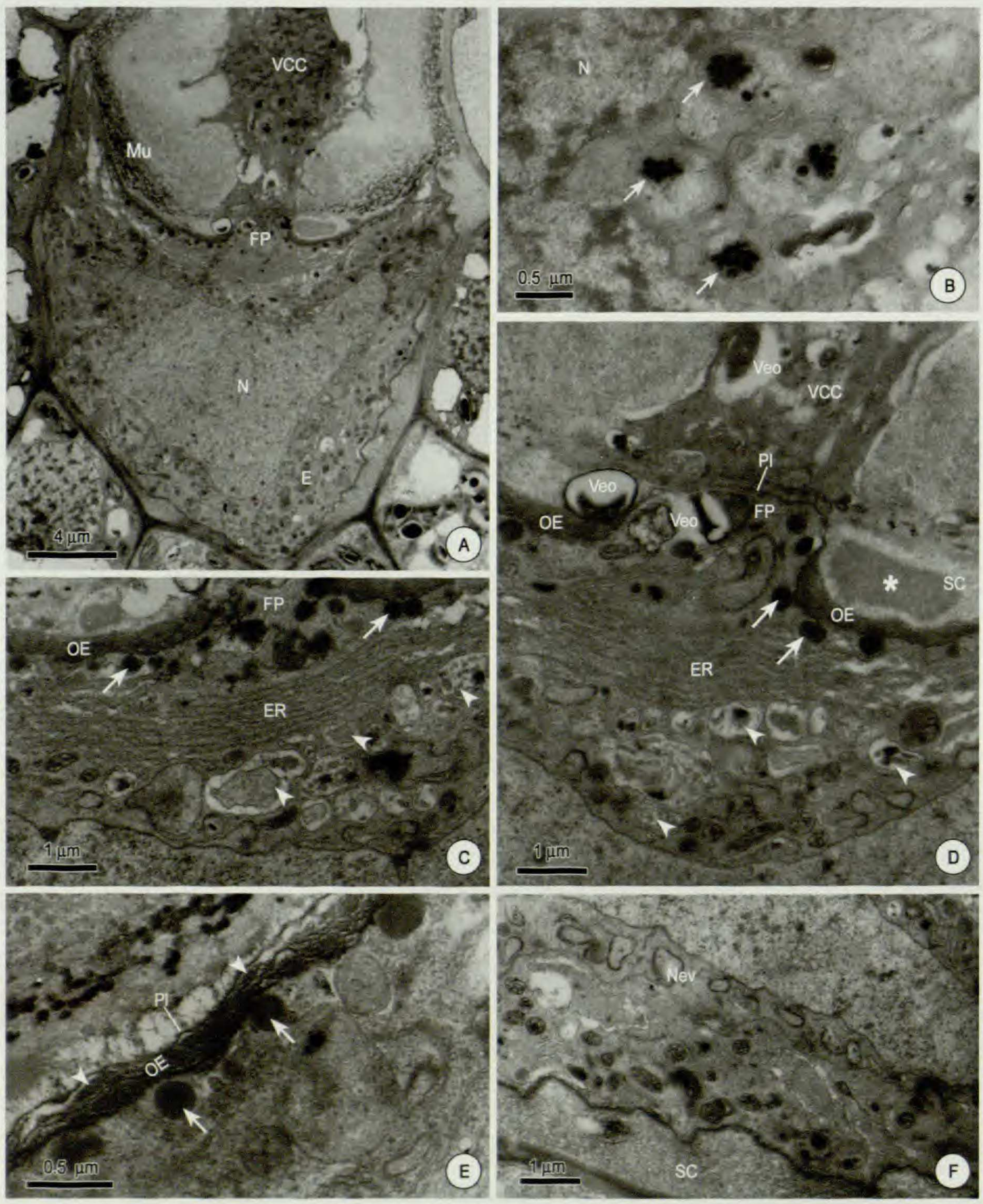

FIG. 5. Mature egg. **A.** A matured egg with a fertilization pore (FP). The VCC is degenerating. **B.** Part of VCC, showing degenerating plastids with clusters of plastoglobuli. **C, D.** Eggs showing pleomorphic vesicles with irregular membrane profiles and osmiophilic deposits at the isolated evagination (arrowheads). Arrows indicate the osmiophilic globules beside the osmiophilic egg envelope (OE). Vesicles containing osmiophilic materials (Veo) near and within the fertilization pore (FP). Electron-opaque materials (asterisk) in the separation cavity (SC). Sheets of endoplasmic reticula are seen in upper part of the egg cytoplasm. **E.** Magnification of the osmiophilic egg envelope showing its fibrillar structure. Osmiophilic globules are arrowed; arrowheads indicate osmiophilic materials within the osmiophilic egg envelope (OE). **F.** Numerous evaginations (Nev) along the lower surface of an egg nucleus. E, Egg; M, mitochondria; N, Nucleus; P, plastid; SC, separation cavity.

obvious beside the osmiophilic egg envelope (Fig. 5C–E, arrows). The egg envelope is composed of a stack of membranes (Fig. 5E). The osmiophilic globules resemble those beside the egg envelope filled in the spaces between membranes of the egg envelope (Fig. 5E, arrowheads). Electron opaque material (asterisk) can be seen in the SC (Fig. 5D).

Discussion

This study shows that the fertilization pore originally described in *Ceratopteris* (Cao *et al.*, 2009) is also formed during egg maturation in *Adiantum*. The transverse diameter of the fertilization pore in *Adiantum* reaches 2.8 μm, slightly larger than that of *Ceratopteris*, which diameter is about 2.5 μm (Cao *et al.*, 2009).

Although a separation cavity had been observed around the egg during oogenesis in a few ferns (Bell and Duckett, 1976; Bao *et al.*, 2003, 2005; Cao *et al.*, 2009), the details of its formation have not been described. The present study shows that the separation cavity first forms along the periphery of the upper surface of the egg. The centripetal expansion of the separation cavity gradually decreases the tissue connection between the egg and the VCC. Our observations confirm previous suggestions that the separation cavity is formed by the elimination of the vesicles from the egg cytoplasm (Bell and Mühlethaler, 1962b; Bell and Duckett, 1976; Bao *et al.*, 2003, 2005). As in *Ceratopteris* (Cao *et al.*, 2009), the absence of the osmiophilic egg envelope in *Adiantum* results in the formation of a fertilization pore.

The VCC almost certainly plays an important role in oogenesis. The persistent plasmodesmatal connections between the VCC and the egg will permit transfer of materials between the two including possible nutrient transfer to the egg (e.g., soluble carbohydrates from the starch in the VCC plastids) as the VCC breaks down. Alternatively the plastid starch may contribute to the mucilage secreted around the VCC from its maturational Golgi activity. In the later stage of the maturing egg, the nucleus of *A. flabellulatum* possesses distinctive evaginations, similar to those in other leptosporangiate ferns so far investigated, including *Pteridium aquilinum* (Bell and Mülethaler, 1962b; Bell and Duckett, 1976), *Dryopteris filix-mas* (L.) Schott (Cave and Bell, 1975), *Histiopteris incisa* (Bell, 1980), *Dryopteris crassirhizoma* (Bao *et al.*, 2005). However, the precise function of these structures, other than a general interpretation that they are a cytological manifestation of profound nucleocytoplasmic interactions preparing the eggs for future growth of the sporophyte (Bell and Duckett, 1976), remains unknown. Although the present study provides no evidence that the evaginations in *Adiantum* become detached as suggested for *Pteridium* (Bell, 1972), we speculate that the evaginations enter into the cytoplasm. The evaginations may eventually be digested in the cytoplasm of the egg as the egg matures, because some of them obviously become vacuolated (Fig. 5C, arrows).

Abundant membranous structures, probably ER, occur in the cytoplasm of the egg, which are also found in the mature eggs of the fern *Ceratopteris*

(Lopez- Smith and Renzaglia, 2008; Cao *et al.*, 2009). The membranous structures, occurring in the cytoplasm of the matured egg of *Adiantum*, may have an analogy to those in the egg of *Ceratopteris*, which is thought to prevent polyspermy (Cao *et al.*, 2009). The occurrence of the membranous structures probably indicates that the egg has become fertilizable.

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